Pancreatic Lipase Hydrolysis of Triglycerides by a Semimicro Technique[1]

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Abstract

Procedures are described for rapid lipase hydrolysis of triglycerides, isolation of the hydrolytic products by TLC and their conversion to methyl esters and fatty acid analysis by GLC. The techniques are applicable to a few mg of triglycerides or fats. Examples of data obtained with purified triglycerides indicate that the specific action of pancreatic lipase for the 1,3 ester groups is nearly absolute and the technique may be used as a criterion of purity of di- and tri-acid triglycerides. Ca. 83% of the palmitic but only 10–12% of stearic and C_{18} unsaturated acids of commercial lard occur in 2-position.

Introduction

THE DISCOVERY that pancreatic lipase preferentially hydrolyzes the fatty acids esterified at the 1,3-positions of triglycerides has stimulated considerable interest in applications to studies of glyceride composition and structure of natural fats. Published work in this area including history of the discovery, factors that influence the rate of lipase hydrolysis, specificity and application to glyceride composition of natural fats have been discussed recently in comprehensive reviews (1,2,2a). The rate and degree of hydrolysis are primarily dependent upon the surface area of the fat substrate produced either by intensity of agitation of the heterogeneous system (or by use of emulsifiers) and at least up to a max, upon the ratio of enzyme to substrate. Presumably the enzyme is adsorbed on the finely dispersed particles of glyceride substrate. Other factors reported to be of importance are pH, presence of electrolytes, bile salts and calcium ions. Digestion temp of ca. 40C are favored. Higher temp increase rate of destruction of enzyme, whereas at lower temp some fats remain as solid or semi-solid, a condition considered less favorable than dispersed liquid particles for efficient adsorption of the lipase. Purified lipase does not hydrolyze esters in solution indicating that lipase action probably takes place at water-oil interfaces.

Until improved techniques for quantitative fractionation and analysis of lipolytic products became available, it was not possible to say how specific was the action of pancreatic lipase for the 1,3-positions of glycerides. However, as these techniques were improved, the more recent work, particularly with pure specimens of triglycerides, suggests that the specificity may be nearly absolute. Mattson and Volpenhein (3) found that some small prcentage of the acids in the 2-position are split off but whether this was due to acyl shifting to the 1-position was unknown Mattson and Beck (4) clearly showed, however, that the monoglycerides isolated from the digestion products were a mixture of 1- and 2-monoglycerides and that increasing proportions of the 1-position were isolated the longer the digestion was allowed to proceed. There is little doubt that this was due to acyl migration from the 2-monoglyceride formed in the selective hydrolysis. Therefore, the fatty acids in the entire monoglyceride fraction may be considered representative of those in the 2-position of the initial triglyceride or fat.

In addition to specificity of the enzyme, however, according to Jack et al. (5), other conditions should be fulfilled in order to apply the principles of the method for quantitative determination of glyceride composition of natural fats: there should be non-preferential hydrolysis of a triglyceride species, absence of substantial amt of complete hydrolysis to free glycerol, absence of significant amt of acyl migration during digestion and no great difference in rates of hydrolysis of various acids in the glycerides.

Evidence of some difference in rates of hydrolysis of acids or triglyceride species has been reported and that simple mono-acid triglycerides such as triolein and trilaurin are hydrolyzed faster than tripalmitin or tristearin (1,5). Some complete hydrolysis, evidenced by free glycerol, also takes places during digestion (5,6,7). Nevertheless, it appears that the extent of these adverse findings has little effect on the fatty acid composition of the monoglycerides produced and does not invalidate the application of the principles of the method.

Procedures have been published for lipase digestion of 100 mg up to 2 g sample and for separating the hydrolytic products by chromatographic columns or countercurrent extraction. The present investigation was undertaken to develop an extensive of the technique to smaller samples such as are often obtained in biochemical investigations and in fractionation of tissue lipids. A procedure is described for extremely rapid hydrolysis of 5–50 mg triglycerides, separation of products by thin-layer chromatography (TLC), conversion of isolated products to methyl esters and their fatty acid analysis by gas-liquid chromatography (GLC). Examples of data obtained and other pertinent observations are reported.

Experimental

The di- and tri-acid triglycerides were synthesized by methods which introduce acyl groups in known positions. The mono-acid triglycerides were ca. 99% pure as determined by GLC on their methyl esters.

Lipolysis of 50 mg Samples. Conditions for lipase hydrolysis of small samples were selected with special attention to extremely rapid agitation in order that the desired degree of hydrolysis could be obtained

TABLE I

Fatty Acid Composition of Triglycerides—GLC

Glyceride[a]	FA, % mol			Impurities, % mol			
	16:0	18:0	18:1	<16:0	16:0	17:0	20:0
POS	33.9	34.1	31.5	....	....	0.6	....
OPS	36.7	33.6	28.5	0.8	....	0.5	....
SOS	.....	66.2	32.1	....	0.7	0.9	....
OSS	.....	67.1	31.5	0.1	0.4	0.4	0.4
Purif. OPS	34.9	32.7	32.4	....	....	....	....
Purif. OSS	.....	66.4	32.9	....	0.3	0.4	....

[a] P = palmitic, O = oleic, S = stearic. The acyl groups are in position indicated. Ex: POS = 2-oleyl 1,3-palmitostearin.

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TABLE II

Lipase Hydrolysis of Glycerides (50 mg) and Analysis of Products

TG	Lipase	Time	% Hydrol.[a]	Products				Fatty acid composiiton,[b] % mol								
								FA			MG			DG		
				FA	MG	DG	TG	16:0	18:0	18:1	16:0	18:0	18:1	16:0	18:0	18:1
	mg	min		mg	mg	mg	mg									
POS	3	0.75	36	17	4	13	13	50	50	tr	2	2	96	26	25	49
POS	3	1.5	41	19	12	11	3	50	50	tr	2	2	96	27	28	45
POS	9	1.5	57	27	16	4	2	49	50	1	1	1	98	27	24	49
POS	9	5.0	60	28	15	4	1	49	50	1	1	2	97	26	27	47
OPS	3	1.5	40	20	6	14	9	11	40	49	97	2	1	51	37	12
OPS	9	1.5	51	25	12	6	2	10	43	47	96	2	2	50	43	7
Purif. OPS	9	1.5	53	26	14	6	2	3	47	50	97	2	1	53	45	2
SOS	3	1.5	27	13	5	15	17	2	97	1	3	7	90	1	51	48
SOS	9	1.5	42	20	11	13	4	1	97	2	2	5	93	1	52	47
SOS	9	5.0	49	23	15	8	2	2	97	1	2	3	95	2	52	46
OSS	3	1.5	27	13	5	14	17	tr	43	57	1	97	2	1	89	10
OSS	9	1.5	40	19	9	12	3	1	41	58	2	97	1	1	94	5
Purif. OSS	9	1.5	45	21	11	10	1	1	42	57	1	99	0	1	97	2
Purif. OSS	9	5.0	51	24	11	6	0	1	46	53	1	98	1	2	96	2

[a] % of theoretical amt of fatty acids possible from wt of sample.
[b] Small amt (<1%) of 17:0 and 16:1 were often observed on GLC. For simplicity of presenting data, the former was included with 18:0, the latter with 18:1.

as quickly as feasible, thereby minimizing acyl migration and destruction of enzyme. Essentially the reagents described by Mattson and Volpenhein (3) were employed, although with some changes in concn in order to have a satisfactory volume for effective agitation. To 50 mg triglyceride weighed in a 5-ml screw-cap vial (45 x 15 mm) was added the predetermined weight of pancreatin (Steapsin). [Nutritional Biochemical Corp. The Steapsin was extracted 2× with acetone (5:1 V/WT) and 2× with anhydrous ethyl ether (5:1) at room temp although no significant differences in results were observed when this extraction was omitted. The amt of pancreatin to use for 50 mg samples depends on the activity of the particular preparation. An amt, usually ca. 9 mg, that gives ca. 50% hydrolysis in 1–2 min is satisfactory.] Then 1 ml 1 M trishydroxymethylaminomethane (adjusted to pH 8), 0.1 ml 22% calcium chloride solution, and 0.25 ml 0.1% bile salts solution were added by means of graduated 1-ml pipettes. The vial and contents were first warmed in a water bath at 40 C for 1 min without shaking. The cap was then screwed on tightly, secured with a strip of plastic tape, and the vial shaken for the time required for 50 (±5)% hydrolysis (usually 1–2 min) in an amalgamator (Model 5-A, Crescent Dental Mfg. Co., Chicago, Ill.). The vial holder was modified and reinforced to accommodate the size vial used. The shaking speed was ca. 3000 strokes/min. The preheating and shaking were carried out in a room regulated at 40C and timed by stopwatch. At the end of the reaction time, contents of the vial were acidified with 0.5 ml 6 N HCl, promptly transferred quantitatively to a small separatory funnel and thoroughly extracted with ethyl ether. The ether extract was washed with distilled water several times until washings were neutral to Congo Red paper, dried over sodium sulfate, filtered and the solvent removed. The weighed residue was transferred quantitatively to a 50-ml volumetric flask with ether, made up to volume and reserved for fractionation and analysis.

Degree of Hydrolysis and Analysis of Fatty Acids. The activities of different lipase preparations varied considerably. Hence it was necessary to determine the ratio of enzyme to sample to employ under the conditions of digestion which gave the desired per cent hydrolysis, i.e., fatty acids liberated expressed as percentage of total possible from sample. It was found that 50 (±5)% is near optimum because in this range the amt of monoglyceride formed was near maximum. The usual titration methods, which are satisfactory for determining percentage of hydrolysis when large samples are employed, were unreliable for small samples. The following procedure was found both convenient and reliable with 5–50 mg samples which had been subjected to lipase digestion. A 5-ml aliquot of the ether solution of hydrolytic products from 50 mg glycerides, or an amt equal to ca. 5 mg, was treated with diazomethane directly to convert the free acids to their methyl esters. A known wt (ca. 1 mg) of internal standard (15:0 methyl ester) was added and the mixture subjected to GLC analysis. Each peak area of the chromatogram was compared to the area for the internal standard which is equal to a known wt, thus permitting calculation of wt and percentages of each component. The sum of wt of all components related to the wt of reaction product (or sample) represented by the aliquot gave the total percentage of free acids. The percentages of the component acids and per cent of hydrolysis found in this way agreed well with those found by direct wt after isolation of free acids by TLC from the remainder of the hydrolytic products. After satisfactory conditions for lipase digestion have been established for the particular lipase preparation, the above technique for determining degree of hydrolysis may be omitted, since the hydrolytic products will then be separated by TLC, and the isolated fatty acids can be weighed.

Lipolysis of 5 mg Samples. The same apparatus and general procedure were employed for 5 mg as for 50 mg with modifications as follows: The pancreatin, 0.9 mg, was added as a freshly made solution in the Tris buffer (1 ml solution of 9 mg in 10 ml Tris). In this way the total amt of buffer was also included. Also added were 0.1 ml 2.2% calcium chloride solution and 0.25 ml 0.05% bile salts solution. The digestion and recovery of products were carried out essentially as described for 50 mg. The recovered

TABLE III

Lipase Hydrolysis of POS (5 and 50 mg) and Analysis of Products

POS	Lipase	Time	% Hydrol.	Products		Fatty acid composition, % mol					
						FA			MG		
				FA	MG	16:0	18:0	18:1	16:0	18:0	18:1
mg	mg	min		mg	mg						
5	0.9	1.5	50	2.5	1.5	49	50	1	3	1	96
50	9	1.5	57	27	16	49	50	1	1	1	98

TABLE IV

Analysis of Monoglycerides and Fatty Acids from Lipase Hydrolysis of Lard (5 and 50 mg)

Lard	Fatty acid composition, % mol						
	<16:0	16:0	18:0	16:1	18:1	18:2	18:3
Triglycerides	1.9	27.8	12.7	3.4	42.6	10.1	1.5
Monoglyc. (2-position) 5 mg [a]	3.7	68.9	5.3	4.3	13.4	4.1	0.3
Monoglyc. (2-position) 50 mg [b]	5.2	68.9	3.8	5.0	13.5	3.0	0.5
% in 2-position avg. [c]	(77.2)	(82.6)	(12.1)	(46.1)	(10.6)	(11.2)	(11.1)
Fatty acids, 5 mg [a]	0.9	7.2	18.3	2.6	57.3	10.6	3.1
Fatty acids, 50 mg [b]	1.0	6.3	18.6	3.0	58.4	10.6	2.1
Fatty acids (1,3) calc [d]	0.7	7.2	16.8	2.7	57.1	13.5	2.0
% in 1,3-position [e]	(22.8)	(17.4)	(87.9)	(53.9)	(89.4)	(88.8)	(88.9)

[a] From lipase hydrolysis of 5 mg lard.
[b] From lipase hydrolysis of 50 mg lard.
[c] % of each fatty acid type in lard glycerides esterified at 2-position calc from mean comp of monoglyceride as described (3).
[d] Fatty acids in 1,3-positions, calc as described (8).
[e] 100—(% in 2-position).

product was made up to ca. 1:10 dilution with chloroform for fractionation by TLC.

Isolation of Lipolytic Products by TLC. Thin-layer plates (20 x 20 cm) were coated singly by spreading a well-stirred mixture of 7 g acetone-washed Silica Gel G and 17 ml distilled water with a spreader designed to give a layer thickness of ca. 275 μ. The plates, after brief air-drying, were activated by heating in an oven at 105C for 2 hr and cooled in a desiccator. The lipolytic product in 1:10 dilution in chloroform was applied dropwise with a microsyringe in a series of spots 1 cm apart. The origin spots were 2.5 cm from the bottom edge of plate. Two chromatoplates were required for the product from digestion of 50-mg samples—one for 5-mg samples. The developing solvent was a mixture of petroleum ether and ethyl ether (60:40) to which 1.6% formic acid was added. All solvents were redistilled before use. The development time was ca. 25 min for the solvent front to reach a height of 120 mm from baseline. The components separated into 4 well-defined zones (rows of spots) in the following order of increasing height travelled: monoglycerides, diglycerides, free acids and unreacted glycerides. The zones were detected (without use of indicator) by holding the chromatoplate just above a white reflector lamp containing 2 (15-watt) fluorescent-light bulbs. When very small amts of sample were chromatographed and the zone spots were indistinct, they were visualized after placing the plate in a tank containing iodine vapor for a few seconds. After marking their location with a needle point the silica gel zones were scraped from the plate into beakers. At this stage the material was handled in 2 ways with equally good results: 1) When 50 mg triglycerides were employed in the lipase digestion, the respective silica gel zones were extracted with ethyl ether, the extracts filtered and residues weighed. Each residue was then converted to methyl esters by acid methanolysis or esterification and fatty acids determined by GLC. 2) When 5 mg triglycerides were employed, each silica gel zone (free acids and monoglyceride) after being scraped from plate was refluxed directly with 10 ml absolute methanol containing 2–3% of concn H_2SO_4 for 1 hr with nitrogen bubbling slowly through the solution to prevent bumping. After the reaction mix was cool, 30 ml petroleum ether was added, the solution filtered into a separatory funnel, washed with water and the petroleum ether solution dried over sodium sulfate. To the dried solution of methyl esters was added a known amt of methyl pentadecanoate (internal standard) estimated to be ca. equal to the expected wt of ester product. The internal standard was added as a 5 ml aliquot of an iso-octane solution of known concn. The solution of methyl esters was then evaporated to dryness and transferred quantitatively with min portions of ethyl ether to a tube, 65 mm x 15 mm, drawn to a sharp cone at one end. The solvent was removed by warming under slow flow of nitrogen, the methyl esters being concn in the tip of the cone, thus facilitating the picking up of a portion in a microsyringe for GLC analysis. The percentage distribution of fatty acids and total wt of the free acid and monoglyceride fractions were calculated from the GLC area relationships and weight of internal standard as described earlier. The purpose in adding the internal standard was to provide a means for calculating the wt of free acids liberated (or percentage hydrolysis) and also of monoglycerides. These wts are not required in calculation of glyceride composition. Therefore, after satisfactory conditions for digestion and desired degree of hydrolysis have been determined, the use of internal standard could be omitted unless the wt of fractions are desired for completeness of data.

Gas-Liquid Chromatography. GLC analyses of methyl esters were performed with an extensively modified commercial apparatus. It was provided with a four-filament thermal conductivity cell detector, and a recorder having a 1 mv, 1-sec full scale pen deflection. The column was an 8 ft x $\frac{3}{16}$ in. OD (ID 0.118 in.) stainless steel coiled tube packed with 42–60 mesh acid and base washed Chromosorb W coated with 25% ethylene glycol succinate polyester. It was heated isothermally at 204C and helium was used as carrier gas. The areas under the peaks of individual components were determined by an electronic integrator coupled to a digital printer. Areas and percentages obtained by this means on known mixtures were found to be in excellent agreement with the known values.

Results and Discussion

The fatty acid composition of the di- and tri-acid triglycerides as received show in Table I. The correspondence with theory mol percentage is reasonably good except for OPS and possibly OSS. Extraneous fatty acid impurities were present up to 1.6%. These glycerides in 50-mg quantities were subjected to li-

TABLE V

Composition of Mixtures of Triglycerides

Glycerides in mixture		Fatty acid in mixture, % (wt)		
	%		Theory [a]	Found [b]
OPS	15.1	M (14:0)	5.3	5.6
POS	20.2	P (16:0)	16.7	16.5
OSS	13.5	S (18:0)	35.8	36.5
SOS	15.0	O (18:1)	42.2	41.4
OOO	20.9			
SSS	5.0			
PPP	4.96			
MMM [c]	5.3			

[a] Theory calculated from known glyceride composition.
[b] Found values determined by GLC on methyl esters.
[c] Trimyristin.

TABLE VI

Lipase Hydrolysis of Known Mixture of Glyceride and Analysis of Products
(50 mg sample, 9 mg lipase, 40C)

Time	% Hydrol.	Products				Fatty acid composition, % wt							
						FA				MG			
		FA	MG	DG	TG	14.0	16:0	18:0	18:1	14.0	16:0	18:0	18:1
		mg	mg	mg	mg								
0.75	54.4	26.9	14.5	6.5	1.9	6.0	14.8	43.7	35.6	6.2	20.2	18.8	54.8
1.5	57.3	28.6	16.9	0.9	1.1	5.9	15.0	44.8	34.3	6.2	20.6	20.0	53.2
					Found avg	6.0	14.9	44.2	35.0	6.2	20.4	19.4	54.0
					Theory, calc[a]	5.3	15.1	44.4	35.2	5.3	20.1	18.5	56.1

[a] Calculated from known composition of mixture.

pase treatment under some variation in time of digestion and amount of lipase. Analysis of the separated products of hydrolysis are given in Table II. The distribution of products with increasing extent of hydrolysis supports previous published observations on the step-wise nature of the hydrolysis and that the rate of hydrolysis of monoglycerides must be very slow compared to that of the triglyceride and diglyceride. It is evident that in general the fatty acid composition of the hydrolytic products conform well with expectations based on specificity of the lipase. Some discrepancies, such as 10–11% palmitic acid in the free acids from OPS, proved to be due to the presence of a small percentage of tri- and dipalmitin as impurity in the sample. Purification of the sample by silicic acid chromatography yielded a product which conformed more nearly with theory in fatty acid composition. Similarly OSS was purified giving a somewhat purer specimen. The analysis of these are included in Table I.

The purified specimens were also digested with lipase and the results are included in Table II. The free acids from purified OPS had much less palmitic acid. The presence of 2–3% monoglyceride impurity in SOS was shown by TLC. This proved to be monostearin after isolation and analysis by GLC, and explains the significant amt of stearic acid found in the monoglycerides reported in the table for SOS. It appears therefore that the specificity under these conditions is nearly-absolute and that the technique can be used as a criterion of purity at least for triglycerides of the higher mol wt acids. There is consistent evidence in the data for OPS and OSS that oleic is hydrolyzed somewhat faster than stearic acid when they are in 1,3-position. The free acids liberated contain excess oleic, resulting in a deficiency of oleic in the diglycerides. This difference in rate, however, does not affect the monoglyceride fatty acids indicating no significant acyl shifting of the 1,2- to the 1,3-diglycerides and subsequent hydrolysis. Coleman (7) also presented evidence indicating that the unsaturated acids hydrolyze faster than higher mol wt saturated acids in work on lard.

Tables III and IV show comparative results obtained on 5- and 50-mg samples of POS and lard on lipase digestion, and separation and analysis of products as described. What differences are noted probably can be attributed to normal experimental error involved in the entire separation and analysis. The results on lard show that ca. 80% of the palmitic and shorter chain saturated acids, mostly myristic, but only 12% of the stearic acid, occur in the 2-position of triglycerides. There appear to be no significant differences in the C_{18} unsaturated acids as far as their occurrence in 2-position, each ca. 10–11%. Palmitoleic, however, is ca. evenly divided between the 2- and 1,3-positions.

Some observations also were made on known mixtures of mono-, di- and tri-acid triglycerides. In one instance, a mixture of 70% triolein and 30% tristearin was subjected to the lipolysis at 40C, yielding free acids containing 94% oleic and only 6% stearic acids. This disparity from the expected was thought to be due to differences in physical state existing in the digestion mixture, most of the tristearin separating as solid aggregates despite the presence of the greater proportion of the liquid triolein. Therefore a more realistic mixture containing 8 triglycerides and lesser proportion of high melting trisaturated glycerides was prepared. The composition as made by direct weighings and, as determined by GLC, shows in Table V.

Two 50-mg portions of the mixture were subjected to lipolysis, the products isolated and analyzed. The results, in Table VI, along with theory values for acids in 1,3- and 2-positions calculated from known composition, show good agreement between the two sets of found values and with theory. The good agreement with theory, particularly for the acids liberated, was somewhat unexpected in view of the presence of ca. 5% each of tristearin, tripalmitin and trimyristin in the mix. Previous observations of our own as well as of others (1,5) indicated significant differences in their rates of hydrolysis compared to triolein which was also present to the extent of 21% in the mixture. Perhaps the explanation is that the melting point of the complex mixture is low enough that all components remain liquid during the digestion and in this state differences in rates of hydrolysis become negligible.

Under the conditions of lipase digestion described, because of very rapid hydrolysis and prompt handling of the products, no appreciable acyl shifting took place in either the mono- or di-glycerides. Analysis of monoglycerides by periodic acid titration from 1.5-min digestions for presence of 1-monoglycerides gave a value of 7.3%; on TLC, only 1 spot for diglycerides was observed. When longer digestions were employed there was increasing evidence of a second spot for 1,3 diglycerides. From the results obtained with the known mixture of glycerides and with the lard sample, there appears little choice in the selection of analysis of monoglycerides over analysis of liberated fatty acids. Perhaps the analysis of both would be desirable as standard procedure.

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